Expanded models for the non-thermal inactivation of *Listeria monocytogenes*

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R.L. BUCHANAN, M.H. GOLDEN AND J.G. PHILLIPS. 1997. Previously developed four-variable response surface models for describing the effects of temperature, pH/lactic acid, sodium chloride and sodium nitrite on the time to achieve a 4-log, non-thermal inactivation (t_{4D}) of *Listeria monocytogenes* in aerobic, acidic environments were expanded to five-variable models that distinguish the effects of pH and acidulant concentration. A total of 18 new variable combinations were evaluated and the inactivation kinetics data appended onto a consolidation of two data sets from earlier studies. The consolidated data set, which included 315 inactivation curves representing 209 unique combinations of the five variables, was analysed by response surface analysis. The quadratic model without backward elimination regression was selected for further evaluation. Three additional quadratic models were generated using the concentrations of undissociated lactic and/or nitrous acids as variables in place of percentage lactic acid and sodium nitrite concentration. Comparison of predicted t_{4D} values against literature values for various food systems indicated that the models provide reasonable initial estimates of the inactivation of *L. monocytogenes*. The models based on the concentration of undissociated lactic and nitrous acids support the hypothesis that antimicrobial activity is associated with this form of the compounds. Evaluation of several examples suggests that these models may be useful for predicting the equivalent of the compounds' 'minimal inhibitory concentrations' for accelerating inactivation under various conditions.

INTRODUCTION

Like all food-borne pathogenic bacteria, the number of viable *Listeria monocytogenes* decreases over time when the micro-organism is placed in an environment that does not support growth. The rate of inactivation is dependent on a variety of factors including pH, acidulant identity, acidulant concentration, water activity, concentration of antimicrobials and temperature (Ahamad and Marth 1989; El-Shenawy and Marth 1989; Parish and Higgins 1989; Sorrels *et al.* 1989; Cole *et al.* 1990; Buchanan *et al.* 1993, 1994; Buchanan and Golden 1994, 1995; Buncic *et al.* 1995). While these factors have been described qualitatively by various investigators, it has only been recently that the interactions among several of the variables have been described quantitatively. This was accomplished through the development of response surface models for the non-thermal inactivation of *L. monocytogenes* as affected by temperature × NaCl × $NaNO_2$ × % lactic acid and temperature × NaCl × $NaNO_2$ × pH under aerobic (Buchanan *et al.* 1994) and oxygen-restricted (Buchanan and Golden 1995) conditions. Although these models are effective, the wide range of buffering capacities encountered in foods and the antimicrobial activity of the lactate anion necessitate development of a model that differentiates the effects of pH and acidulant concentration. Accordingly, additional experimental data were generated and new aerobic models, reported here within, were generated. Models were also generated to evaluate the use of calculated levels of

undissociated lactic acid and/or nitrous acid in place of total lactic acid or sodium nitrite.

MATERIALS AND METHODS

With the exception of the adjustment of the final pH of the experimental cultures, the techniques employed are the same as those of Buchanan *et al.* (1994) and Buchanan and Golden (1995). These techniques are described briefly below.

Micro-organisms

A three-strain mixture of *Listeria monocytogenes* (Scott A, HO-V-S and V-7) was used. These strains were originally isolated from a clinical, ground beef and milk sample, respectively. The strains were cultured individually in Brain Heart Infusion broth (BHI) (Difco, Detroit, MI) for 24 h at 37°C and then combined to produce an inoculum of $\approx 10^9$ cfu ml^{-1}.

Test system

Double-strength BHI was supplemented with crystalline sodium chloride and 85% lactic acid to achieve the desired concentrations (0·5–19·0% and 0·0–2·0%, respectively) and then brought up to volume. The pH was then adjusted to the target level using a 50% NaOH solution or 10 mol l^{-1} HCl. After recording the pH, the medium was dispensed in 19-ml portions into milk dilution bottles and autoclaved. Immediately prior to inoculation, filter-sterilized sodium nitrite was added to achieve concentrations of 0–200 μg ml^{-1}. The volume of the sodium nitrite solution and the inoculum (see below) was taken into account in the preparation of the medium to achieve a final volume in each bottle of 20 ml.

Inactivation studies

Each bottle was inoculated with 0·6 ml of the diluted three-strain mixture to achieve an initial population density of $\approx 10^8$ cfu ml^{-1}. The cultures were incubated aerobically without agitation at 4°, 12°, 19°, 28°, 37° or 42°C. Periodically, the cultures were agitated and 0·1 ml samples were removed, diluted appropriately in 0·1% peptone water, and surface plated on tryptose agar (Difco) using a Spiral Plater (Spiral Systems, Inc., Cincinnati, OH) or spread plates (at lower population densities). All plates were incubated for 24 h at 37°C. Sampling continued for 6 months or until the counts fell below the lower limit of detection ($\log_{10} < 1{\cdot}03$ cfu ml^{-1}). With the exception of verifying that the pH of the BHI had changed by $\leqslant 0{\cdot}1$ pH unit after autoclaving, no attempt was made to follow changes in pH, residual $NaNO_2$ or % NaCl.

Survivor curves/primary models

Survivor curves were generated by fitting the data to the linear primary model of Buchanan *et al.* (1993).

$$Y = Y_o \quad [t \leqslant t_L]$$

$$Y = Y_o + s(t - t_L) \quad [t > t_L]$$

where $Y = \log_{10}$ count at time t ($\log_{10}$(cfu ml^{-1})), $Y_o = \log_{10}$ count at time $t = 0$ ($\log_{10}$(cfu ml^{-1})), s = slope of the survivor curve ($\log_{10}$(cfu ml^{-1}) h^{-1}), t = time (h) and t_L = duration of lag period (h).

D-values were calculated by taking the negative reciprocal of the s-value. The 'time to a 4-D (99·99%) inactivation' (t_{4D}) was calculated using the equation:

$$t_{4D} = t_L + (4D).$$

Secondary models

The data generated were appended onto the previously generated aerobic data sets (Buchanan *et al.* 1993, 1994; Buchanan and Golden 1994). This consolidated data set was then submitted to response surface analysis to generate secondary models of t_{4D} values on the independent variables (SAS Institute Inc. 1989) after a natural logarithmic transformation of the t_{4D} data was used to stabilize the variance. Quadratic and cubic polynomials were generated. The analyses were also submitted to stepwise backward elimination regression analysis. A probability of $< 0{\cdot}1$ was used as the retention criterion for that analysis. The five-variable models generated included:

- temperature × NaCl × $NaNO_2$ × % lactic acid × pH,
- temperature × NaCl × $NaNO_2$ × undissociated lactic acid × pH,
- temperature × NaCl × undissociated HNO_2 × % lactic acid × pH,
- temperature × NaCl × undissociated HNO_2 × undissociated lactic acid × pH.

The concentrations of undissociated lactic and nitrous acids were calculated using pK_a values of 3·86 and 3·40, respectively. Matrices for calculating 95% confidence intervals associated with the models' predictions were used to estimate specific values of the five variables (Draper and Smith 1989).

RESULTS

Eighteen variable combinations for which the final pH was adjusted to a predetermined value after the addition of lactic acid were evaluated under aerobic conditions (Table 1). A similar set of data (not shown) was also obtained for the same variable combinations tested under oxygen-restricted conditions. The similarity of responses for aerobic and anaerobic conditions noted in the current and earlier studies (Buch-

Table 1 Inactivation kinetics data observed for 18 new variable combinations (these data were added to existing aerobic data sets (see text))

Temperature (°C)	Lactic acid (%)	NaCl (%)	$NaNO_2$ ($\mu g\ ml^{-1}$)	pH	N_o (log [cfu m^{-1}])	t_L (h)	Slope ([log (cfu ml^{-1})] h^{-1})	D-value (h)	t_{4D} (h)
42	0·0	0·5	0	4·0	7·7	13·4	−0·085	11·75	60·4
42	0·5	0·5	50	4·5	7·8	1·0	−0·152	6·58	27·3
37	1·0	10·3	200	5·0	7·7	1·0	−0·297	3·37	14·5
28	0·0	0·5	200	4·5	7·6	7·0	−0·047	21·32	92·3
28	1·5	0·5	100	4·0	7·7	0·0	−0·878	1·14	4·6
28	1·0	7·8	150	5·0	7·7	7·0	−0·049	20·62	89·5
19	0·0	0·5	200	4·0	7·7	1·0	−0·097	10·27	42·1
19	2·0	2·5	25	3·0	7·5	0·0	−0·814	1·23	4·9
19	2·0	19·0	0	3·5	7·5	0·0	−0·348	2·87	11·5
19	1·0	19·0	200	5·0	7·6	0·3	−0·044	22·48	90·2
12	1·5	4·5	100	3·5	8·0	0·0	−2·165	0·46	1·9
12	2·0	19·0	200	6·0	7·9	414·6	−0·005	213·10	1267·0
12	2·0	19·0	100	5·0	7·9	0·0	−0·019	53·62	214·5
4	1·0	0·5	100	3·0	7·8	0·0	−2·378	0·42	1·7
4	1·0	4·5	200	4·0	8·0	1·0	−0·167	6·01	24·0
4	1·0	7·8	150	3·5	7·9	0·0	−2·291	0·44	1·8
4	2·0	19·0	200	3·2	7·9	0·0	−7·361	0·14	0·5
4	1·0	19·0	100	3·0	7·9	0·0	−7·066	0·14	0·6

See text for abbreviations.

anan and Golden 1995), along with the greater availability of aerobic data, led us to focus on the development of aerobic models. The additional aerobic data were appended to the data sets generated previously (Buchanan *et al.* 1993, 1994; Buchanan and Golden 1994) to provide a total of 315 aerobic cultures representing 209 unique combinations of the five variables.

The Ln-transformed t_{4D} data were submitted to response surface analysis using both quadratic and cubic polynomial models. The models were also analysed using stepwise backward elimination regression to remove non-significant terms. Comparison of R^2 values (Table 2) indicated that all of the models gave reasonable fits with the experimental data. The fits were enhanced only to a relatively small degree by the use of cubic models. The submission of the models to stepwise backward elimination regression had minimal effect on R^2 values.

While the cubic models provided somewhat higher R^2 values, comparison of the predictions provided by the quadratic and cubic models for temperature × pH × % lactic acid × sodium nitrite × sodium chloride identified anomalies in the cubic models in relation to the interaction of temperature, pH and lactic acid concentration. Specifically, while the quadratic model predicts that *L. monocytogenes* would be inac-

Table 2 R^2 values for the various models generated by response surface analysis with and without stepwise backward elimination regression

Model	Variables			
	T × S × P × L × N	T × S × P × H_L × N	T × S × P × L × H_N	T × S × P × H_L × H_N
Quadratic/full regression	0·874	0·871	0·861	0·859
Quadratic/backward regression	0·870	0·870	0·859	0·856
Cubic/full regression	0·904	0·904	0·899	0·900
Cubic/backward regression	0·899	0·897	0·894	0·892

T, Temperature (°C); S, sodium chloride (%); P, pH; L, lactic acid (%); N, sodium nitrite ($\mu g\ ml^{-1}$); H_L, undissociated lactic acid (mmol l^{-1}); H_N, undissociated nitrous acid ($\mu mol\ l^{-1}$).

tivated more rapidly with increasing lactic acid concentration at both high and low storage temperatures (e.g. 4°C *vs* 30°C), the cubic model predicted that lactic acid provides a substantial protective effect at the lower temperature, particularly at higher pH values. Examination of the experimental data indicated that the cubic models' anomalies are likely due to a paucity of data at the lower temperatures.

It is likely that the anomaly in the cubic model could be corrected by the acquisition of additional data. However, it was decided not to further pursue the cubic models and to concentrate on the quadratic models. Further evaluation indicated that both the full and stepwise backward elimination quadratic models predict that at the higher pH values (pH $\geqslant$ 6·0), high levels of sodium nitrite provide a small protective effect. An example (20°C, 0·5% NaCl, 0·3% lactic acid) of the predicted interaction between pH and sodium nitrite concentration is depicted in Fig. 1. It is not uncommon to get this type of curvilinear response at the extremes of the variable ranges when fitting models using response surface techniques. This is one of the reasons for selecting variable ranges that extend beyond the range of interest (i.e. sodium nitrite levels up to 200 μg g^{-1}). In practice, this deviation should have little impact on the usefulness of the model since it occurs at sodium nitrite concentrations > 150 μg ml^{-1}, values well beyond the residual sodium nitrite levels that would be encountered in cured meat products. In general, the predicted protective effect of high sodium nitrite levels was greater for the backward elimination regression models. Because of this and the small number of terms removed by the backward elimination process, the full quadratic model (model 1) (Table 3) was selected for further evaluation.

A scatter diagram comparing the fit provided by the temperature × pH × % lactic acid × sodium nitrite × sodium chloride model between predicted and observed t_{4D} values for the 315 experimental trials is provided in Fig. 2. While

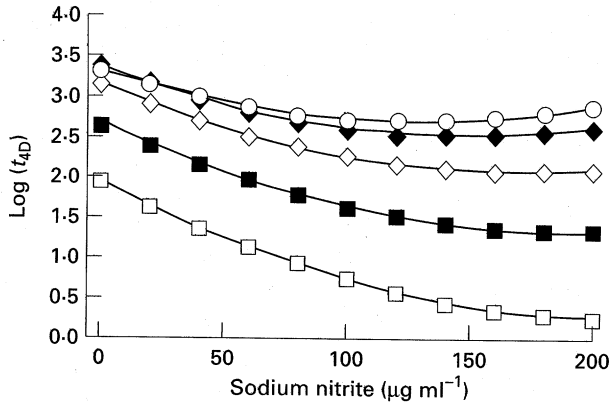

Fig. 1 Example (20°C, 0·5% NaCl, 0·3% lactic acid) of the effects of pH and sodium nitrite concentration on the inactivation of *Listeria monocytogenes* as predicted by model 1 (Table 3). pH: □, 3·0; ■, 4·0; ◇, 5·0; ◆, 6·0; ○, 7·0

Table 3 Model 1: full quadratic response surface model for the effects of temperature, pH, lactic acid content, sodium chloride content and sodium nitrite concentration on the 'time to achieve a 4-D' (t_{4D}) inactivation of *Listeria monocytogenes*

Variables:
T = temperature (4–42°C)
S = sodium chloride (0·5–19·0% aqueous phase)
P = pH (3·2–7·3)
L = lactic acid (0–2% w/w)
N = sodium nitrite (0–200 μg ml^{-1})

$$\begin{aligned}\text{Ln}(t_{4D}) = {} & -3{\cdot}666+0{\cdot}0371\text{T}+0{\cdot}0575\text{S}+3{\cdot}9024\text{P}-1{\cdot}749\text{L} \\ & -0{\cdot}0547\text{N}-0{\cdot}0012\text{TS}-0{\cdot}00812\text{TP}-0{\cdot}0131\text{TL} \\ & +0{\cdot}000323\text{TN}+0{\cdot}0103\text{SP}+0{\cdot}00895\text{SL}+0{\cdot}000152\text{SN} \\ & +0{\cdot}1895\text{PL}+0{\cdot}00356\text{PN}+0{\cdot}00209\text{LN}-0{\cdot}00168\text{T}^2 \\ & -0{\cdot}00749\text{S}^2-0{\cdot}3007\text{P}^2+0{\cdot}1705\text{L}^2+0{\cdot}0000871\text{N}^2\end{aligned}$$

there was a substantial amount of variation, overall the fit was good, particularly considering that the t_{4D} values range from minutes to months. A maximum predicted t_{4D} value of 4300 h was imposed, corresponding approximately to the maximum duration of the experimental trials.

In addition to direct pH effects, weak organic acids like lactic acid have antimicrobial activity that is hypothesized to be dependent on their presence as undissociated acids (Grau 1981; Gill and Newton 1982; Buchanan *et al.* 1993). Similarly, the antimicrobial activity of sodium nitrite is hypothesized to be dependent on its conversion to undissociated nitrous acid (Castellani and Niven 1955). The current data set provides an extensive body of experimental values for assessing the relationship between bacterial inactivation kinetics and the concentrations of undissociated lactic and nitrous acids. Potentially, such data could lead to insights concerning the physiological basis for the compounds' antimicrobial activity.

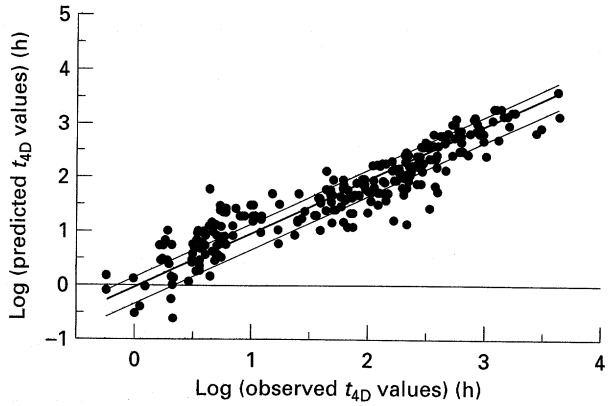

Fig. 2 Scatter diagram of t_{4D} values and those predicted by model 1 (Table 3). The centre line is the 'line of equivalence' and the two outside lines are ± 50% of observed t_{4D} value

Accordingly, the concentrations of undissociated lactic and nitrous acids were calculated for each of the experimental cultures. The data were then reanalysed to develop three additional sets of models wherein the concentrations of undissociated lactic acid (model 2, Table 4), nitrous acid (model 3, Table 5) and lactic + nitrous acids (model 4, Table 6) were considered as variables.

The acquisition of a limited set of additional experimental data allowed the effects of pH and acidulant concentration to be differentiated sufficiently to successfully expand the previous models for the non-thermal inactivation of *L. monocytogenes* to simultaneously include five variables: temperature, pH, and the concentrations of lactic acid, sodium nitrite and sodium chloride (Table 3). This also allowed additional models to be developed based on the concentrations of undissociated lactic and/or nitrous acids (Tables 4–6). These additional models should be helpful for exploring the physicochemical basis underlying the response of *L. monocytogenes* to the environmental variables. For example, model 2 (Table 4) was used to predict the effects and interaction of pH (3·0–6·5) and lactic acid concentration (0–2%), with temperature, NaCl and $NaNO_2$ being held constant at 25°C, 1·5% and 0 μg ml^{-1}, respectively (Fig. 3). When the total concentration of lactic acid was considered (Fig. 3a), the pH-dependent nature of the acid's activity against *L. monocytogenes* is readily apparent. The greatest absolute enhancement of *L. monocytogenes* inactivation with increasing lactic acid concentration is predicted to occur in the pH range of 4·0–5·0, while little activity is predicted at pH $\geqslant$ 5·5. When the predicted t_{4D} values are expressed on a logarithmic scale (Fig. 3b), it becomes more apparent that the greatest relative impact associated with lactic acid anion effects is predicted to occur at pH values $<$ 4·0. However, on an absolute basis this is masked by the strong effect that pH alone has on *L. monocytogenes* survival. When the concentration of undissociated lactic acid was considered (Fig. 3c), plotting the log of the t_{4D} values *vs* the logarithm of the undissociated lactic acid concentration yields a family of curves that suggest that there is a minimum concentration of undissociated lactic acid ($\approx$ 3–6 mmol l^{-1}) that must be reached before the acid produces distinct anion effects. At the higher pH levels, this minimum concentration of undissociated acid would not be reached even at the 2% lactic acid level. Because the logarithm of zero is undefined, in Fig. 3c the effect of pH alone on predicted inactivation rates was approximated by assigning these t_{4D} values an arbitrary low undissociated lactic acid concentration of 0·01 mmol l^{-1}. On an undissociated acid basis, the model correctly predicts that the greatest enhancement of inactivation occurs at the lowest pH, where the

Table 4 Model 2: full quadratic response surface model for the effects of temperature, pH, sodium chloride content, sodium nitrite concentration and calculated concentration of undissociated lactic acid on the 'time to achieve a 4-D' (t_{4D}) inactivation of *Listeria monocytogenes*

Variables:
T = temperature (4–42°C)
S = sodium chloride (0·5–19·0% aqueous phase)
P = pH (3·2–7·3)
H_L = undissociated lactic acid (mmol l^{-1})
N = sodium nitrite (0–200 μg m^{-1})

$$\begin{aligned}\text{Ln}(t_{4D}) = {} & -0{\cdot}7931+0{\cdot}0129T+0{\cdot}0536S+2{\cdot}8624P-0{\cdot}0207H_L \\ & -0{\cdot}556N-0{\cdot}00125TS-0{\cdot}00479TP-0{\cdot}0000579TH_L \\ & +0{\cdot}000325TN+0{\cdot}0136SP+0{\cdot}000102SH_L+0{\cdot}000118SN \\ & +0{\cdot}000682PH_L+0{\cdot}00383PN+0{\cdot}0000308H_LN-0{\cdot}00162T^2 \\ & -0{\cdot}00761S^2-0{\cdot}2182P^2+0{\cdot}0000378H_L^2+0{\cdot}0000879N^2\end{aligned}$$

Table 5 Model 3: full quadratic response surface model for the effects of temperature, pH, sodium chloride content, lactic acid content and calculated concentration of undissociated nitrous acid on the 'time to achieve a 4-D' (t_{4D}) inactivation of *Listeria monocytogenes*

Variables:
T = temperature (4–42°C)
S = sodium chloride (0·5–19·0% aqueous phase)
P = pH (3·2–7·3)
L = lactic acid (0–2%)
H_N = undissociated nitrous acid (μmol l^{-1})

$$\begin{aligned}\text{Ln}(t_{4D}) = {} & 0{\cdot}7511-0{\cdot}0163T+0{\cdot}0121S+2{\cdot}3595P-1{\cdot}888L \\ & -6{\cdot}2374H_N-0{\cdot}00108TS+0{\cdot}00725TP-0{\cdot}00496TL \\ & +0{\cdot}0731TH_N+0{\cdot}0139SP+0{\cdot}00688SL+0{\cdot}0431SH_N \\ & +0{\cdot}2035PL-0{\cdot}6851PH_N+0{\cdot}9209LH_N-0{\cdot}00195T^2 \\ & -0{\cdot}00637S^2-0{\cdot}2013P^2+0{\cdot}1459L^2+2{\cdot}3993H_N^2\end{aligned}$$

Table 6 Model 4: full quadratic response surface model for the effects of temperature, pH, sodium chloride content and the calculated concentrations of undissociated lactic and nitrous acid on the 'time to achieve a 4-D' (t_{4D}) inactivation of *Listeria monocytogenes*

Variables:
T = temperature (4–42°C)
S = sodium chloride (0·5–19·0% aqueous phase)
P = pH (3·2–7·3)
H_L = lactic acid (mmol l^{-1})
H_N = undissociated nitrous acid (μmol l^{-1})

$$\begin{aligned}\text{Ln}(t_{4D}) = {} & 3{\cdot}6255-0{\cdot}0226T-0{\cdot}015S+1{\cdot}1417P-0{\cdot}0192H_L \\ & -7{\cdot}2762H_N-0{\cdot}000879TS+0{\cdot}00779TP-0{\cdot}000471TH_L \\ & +0{\cdot}0719TH_N+0{\cdot}0164SP+0{\cdot}000164SH_L+0{\cdot}0465SH_N \\ & +0{\cdot}00256PH_L-0{\cdot}4202PH_N+0{\cdot}0106H_LH_N-0{\cdot}0019T^2 \\ & -0{\cdot}00618S^2-0{\cdot}0874P^2+2{\cdot}5125H_N^2\end{aligned}$$

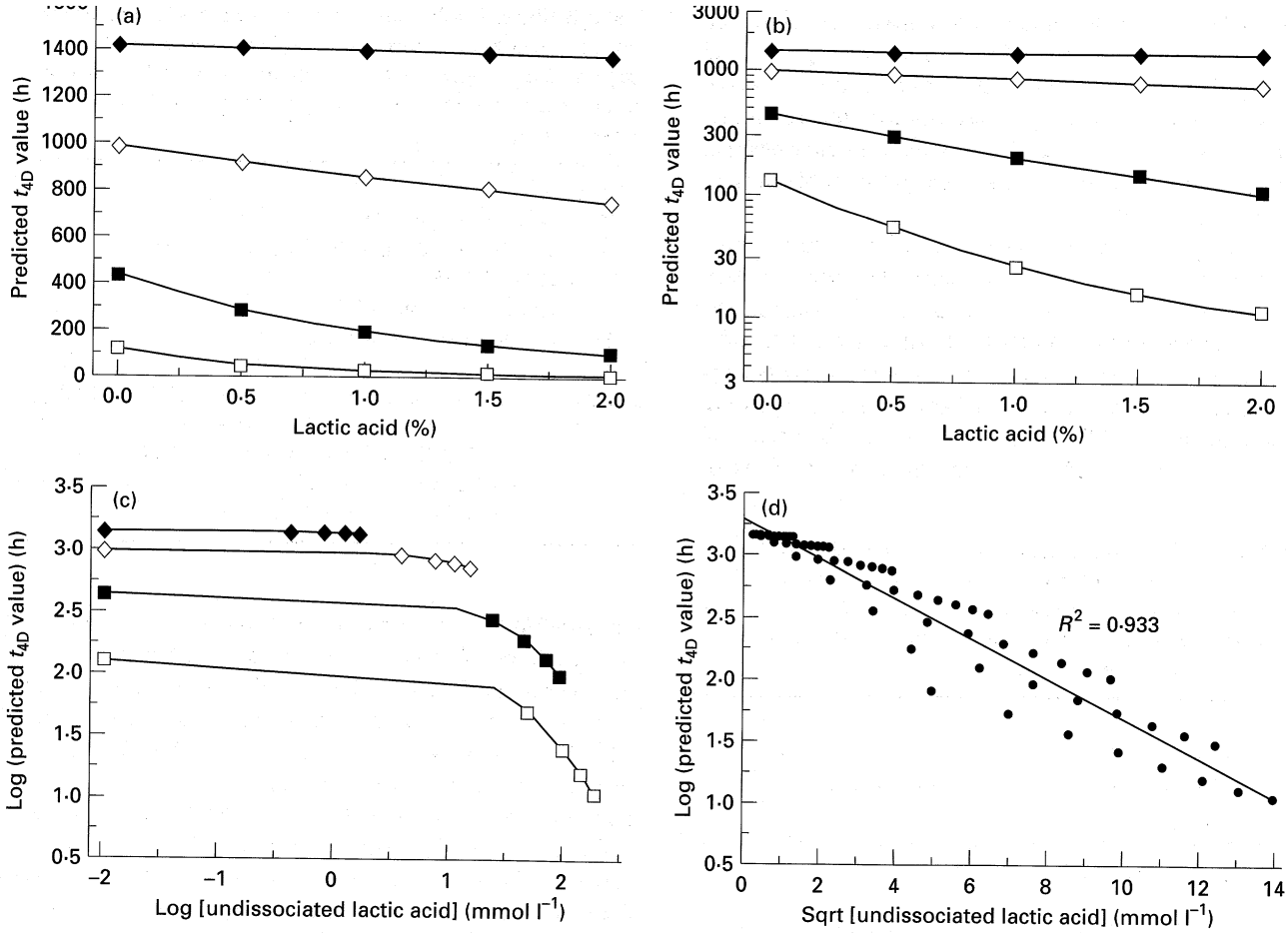

Fig. 3 Example (25°C, 1·5% NaCl, 0 μg ml^{-1} $NaNO_2$) of the effects of pH and lactic acid concentration on the inactivation of *Listeria monocytogenes* as predicted by model 2 (Table 4). (a) Total lactic acid *vs* t_{4D}. (b) Total lactic acid *vs* t_{4D} on a logarithmic scale. (c) Log (undissociated lactic acid) *vs* log (t_{4D}). Lactic acid-free values arbitrarily assigned an undissociated acid concentration of 0·01 mmol l^{-1}. (d) Square root (undissociated lactic acid) *vs* log (t_{4D}). Lactic acid-free values excluded. Additional pH values (3·5, 4·5, 5·5 and 6·5) and lactic acid values included in evaluation of predicted responses. pH: □, 3·0; ■, 4·0; ◇, 5·0; ◇, 6·0

greater portion of the lactic acid would be present in its undissociated form. The minimum concentration of undissociated lactic acid needed to accelerate the inactivation of *L. monocytogenes* in the current example is somewhat greater than the 1·8–2·2 mmol l^{-1} minimal inhibitory concentration (MIC) calculated from the results of Houtsma *et al.* (1993) for the inhibition of growth at pH 6·5. This difference likely reflects differences inherent in the two physiological processes (i.e. inactivation *vs* bacteriostasis). In addition, the MIC reported by Houtsma *et al.* (1993) is confounded to an unknown degree by the water activity lowering effect associated with the high sodium lactate concentration (equivalent to 7–8% lactic acid) that had to be employed to prevent growth.

In an earlier study of the effects of pH and lactic or acetic acid concentration on the inactivation of *L. monocytogenes*, it was observed that for the single set of cultural conditions examined, the logarithm of the t_{4D} values for organic acid-containing cultures was linearly related to the square root of the undissociated acid concentration (Buchanan *et al.* 1993). Interestingly, after excluding the lactic acid-free cultures, the current example predicts a similar linear (R^2 = 0·933) relationship between calculated undissociated lactic acid concentration and predicted t_{4D} values (Fig. 3c):

$$\text{Log}\,[t_{4D}] = -0{\cdot}15994(H_L)^{0{\cdot}5} + 3{\cdot}28542.$$

It appears that the previously observed linear relationship (Buchanan *et al.* 1993) gives an approximation of the family of pH/undissociated acid curves described by model 2 (Fig. 3c). The physicochemical basis for why this specific relationship provides a linear estimation of the combined effects of pH and acidulant concentration requires further elucidation.

Model 3 (Table 5) was used to explore the dependency of *L. monocytogenes* inactivation rates on the interaction of pH

(3·0–6·5) and sodium nitrite concentration (0–150 $\mu g\ g^{-1}$) (Fig. 4). In this example, temperature, NaCl level and percentage lactic acid were held constant at 15°C, 3·5% and 0·5%, respectively, to approximate conditions that might be encountered with a fermented meat product. As was observed with lactic acid, the model predicts that sodium nitrite has its greatest absolute enhancement of *L. monocytogenes* inactivation in the pH range 4·0–5·0, with relatively little activity at pH $\geqslant$ 5·5 (Fig. 4a). Again, the greatest relative activity is predicted at the lowest pH values (Fig. 4b). When the log of the predicted t_{4D} values was plotted against the log of the concentration of undissociated nitrous acid (Fig. 4c), a family of pH/nitrous acid curves was obtained that was similar in general characteristics to that noted for pH/lactic acid (Fig. 3c). Again, due to the undefined nature of the logarithm of zero, the effect of pH alone was approximated graphically by depicting an arbitrary low undissociated nitrous acid value of 10^{-5} $\mu mol\ ml^{-1}$. The model suggests that under the conditions used in this example, the minimum level of undissociated nitrous acid needed to accelerate *L. monocytogenes* inactivation is $\approx 0{\cdot}1$ $\mu mol\ ml^{-1}$.

The similarity in the shape of the family of pH/undissociated acid curves predicted for both lactic and nitrous acids prompted an assessment of whether the linear relationship between the log of t_{4D} values and the square root of undissociated acid held true for sodium nitrite (Fig. 4d). It is evident that after excluding the values for nitrite-free cultures there was a strong linear correlation ($R^2 = 0{\cdot}978$) between the undissociated nitrous acid concentration and inactivation rate:

$$\text{Log}\,[t_{4D}] = -2{\cdot}98929(HNO_2)^{0{\cdot}5} + 3{\cdot}38878.$$

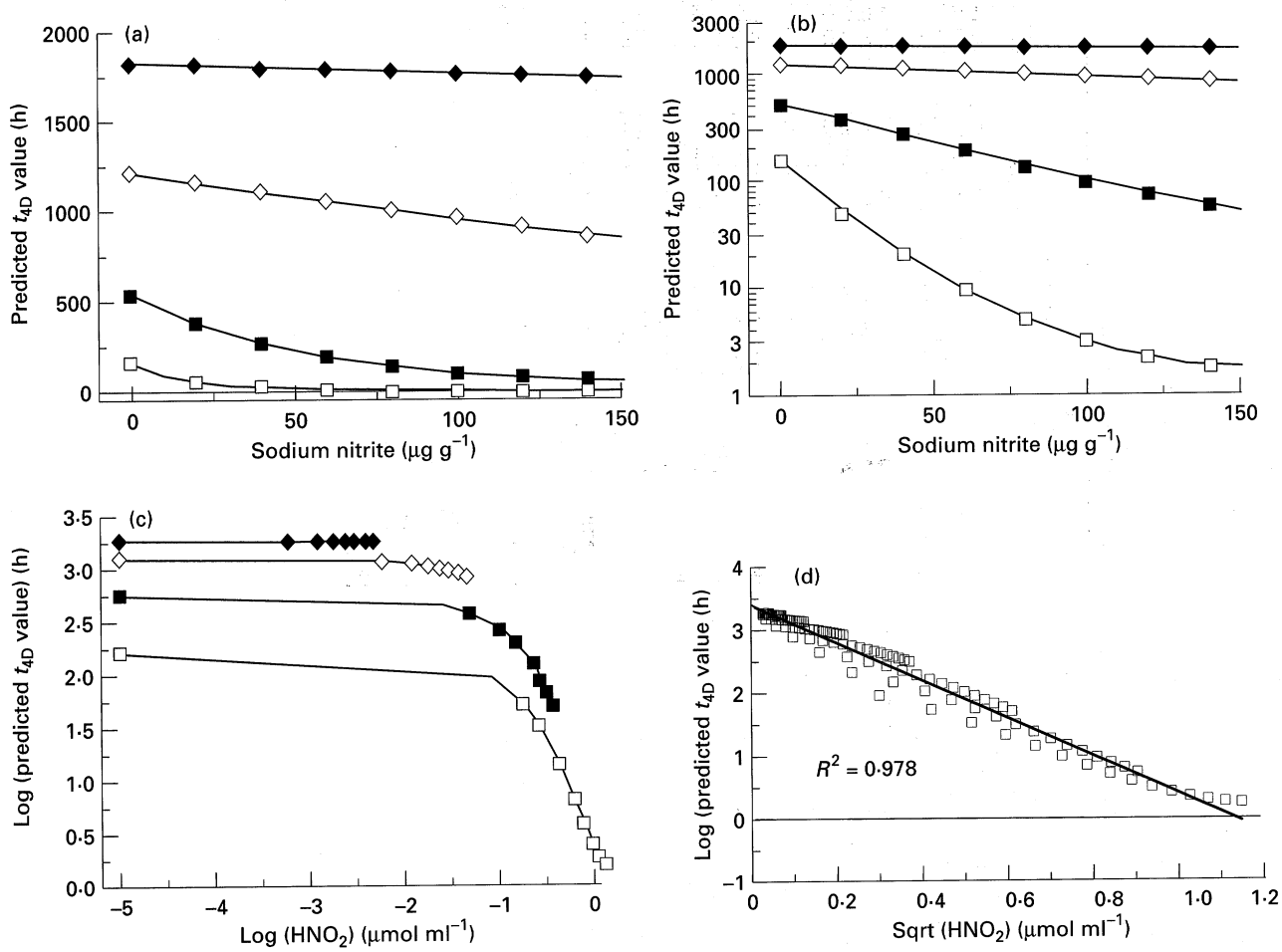

Fig. 4 Example (15°C, 3·5% NaCl, 0·5% lactic acid) of the effects of pH and sodium nitrite/undissociated nitrous acid concentration on the inactivation of *Listeria monocytogenes* as predicted by model 3 (Table 5). Symbols are the same as those in Fig. 3. (a) Sodium nitrite *vs* t_{4D}. (b) Sodium nitrite *vs* t_{4D} on a logarithmic scale. (c) Log (undissociated nitrous acid) *vs* log (t_{4D}). Nitrite-free values arbitrarily assigned an undissociated nitrous acid concentration of 10^{-5} $\mu mol\ ml^{-1}$. (d) Square root (undissociated nitrous acid) *vs* log (t_{4D}). Nitrite-free values excluded. Additonal pH values (3·5, 4·5, 5·5 and 6·5) and nitrous acid concentrations included in evaluation of predicted responses

Table 7 Comparison of literature values with those predicted by inactivation models

Product	Temperature (°C)	NaCl (%)	$NaNO_2$ ($\mu g\ g^{-1}$)	Lactic acid (%)	pH	Strain(s)	Estimated t_{4D} from literature (h)	Predicted t_{4D} value (h) Model no. 1	Model no. 2	Model no. 3	Model no. 4	Reference	Comments
Unclarified cabbage juice	4	NR*	NR*	NR*	5·6	LCDC81–86	3460	4567 (2485–8394)	4256 (2331–7773)	1654 (901–3035)	1568 (866–2836)	Conner *et al.* 1986	*Assumed NaCl = 0·5%, lactic acid = 0·5%, $NaNO_2$ = 0
		1·0				LCDC81–86	3340	4811 (2656–8121)	4505 (2499–8712)	1721 (953–3108)	1618 (907–2887)		
		4·5				Scott A	6340	6024 (3541–10247)	6024 (3541–10247)	2076 (1241–3475)	1857 (1113–3099)		
		4·5				LCDC81–86	2020	6230 (3697–10501)	6024 (3541–10247)	2076 (1241–3475)	1857 (1113–3099)		
Clarified cabbage juice	4	NR*	NR*	NR*	5·6	LCDC81–86	8000	4567 (2485–8394)	4256 (2331–7773)	1654 (901–3035)	1568 (866–2836)	Conner *et al.* 1986	*Assumed NaCl = 0·5%, lactic acid = 0·5%, $NaNO_2$ = 0
	30						238**	689 (504–944)	697 (523–929)	515 (370–716)	498 (369–673)		**Growth followed by inactivation
	4				4·8		2670	2327 (1444–3751)	2462 (1496–4051)	1198 (751–1910)	1218 (755–1967)		
	30						336	416 (323–535)	442 (347–563)	321 (247–417)	327 (254–421)		
	4				4·4		202	1438 (920–2247)	1563 (979–2497)	926 (595–1439)	987 (626–1555)		
	30						110	279 (222–352)	292 (233–366)	230 (182–291)	242 (192–306)		
Tryptic soy broth	35	10	0	0	4·8	H4	613*	370 (264–519)	264 (190–366)	238 (168–335)	146 (107–200)	Sorrells and Enigl 1990	*Based on estimates of initial and final populations only
	25	12			4·8		870	980 (718–1337)	745 (541–1025)	592 (431–814)	357 (265–481)		
	10	10			5·2		2800	5020 (3412–7386)	3895 (2665–5692)	2188 (1499–3193)	1311 (927–1854)		
	10	8			5·0		4560	4559 (3128–6642)	3562 (2490–5095)	2155 (1496–3105)	1321 (954–1832)		
	35	10			4·8	31C	646	370 (264–519)	264 (190–366)	238 (168–335)	146 (107–200)		
	25	12			4·8		1050	980 (718–1337)	745 (541–1025)	592 (431–814)	357 (265–481)		
	10	10			5·2		1690	5020 (3412–7386)	3895 (2665–5692)	2188 (1499–3193)	1311 (927–1854)		
	10	8			5·0		1200	4559 (3128–6642)	3562 (2490–5095)	2155 (1496–3105)	1321 (954–1832)		
Tryptic soy broth + 0·6% yeast extract	4	0·5	0	0	4·5	V-37, N7183, 47045, N7095	1050	2514 (1409–4485)	2126 (1281–3527)	1570 (883–2791)	1136 (698–1848)	Conner *et al.* 1990	
Salami	13	5·9	50*	NR*	4·3	Scott A	273	371 (292–471)	293 (228–376)	478 (392–582)	467 (378–576)	Johnson *et al.* 1988	Held at 40°C for 24 h, and then lowered to 13°C *Assumed 30% residual of initial 156 $\mu g\ g^{-1}$ $NaNO_2$ *Assumed 0·5% lactic acid
Fermented beaker sausage	32	3·3	50*	NR*	4·5	Scott A, V7, LM101M, LM102M, LM103M	124	70 (55–90)	96 (73–125)	161 (131–198)	163 (132–201)	Glass and Doyle 1989	Initial pH of 6·0 fell to 4·8 within 12 h during fermentation *Assumed 30% residual of initial 156 $\mu g\ g^{-1}$ $NaNO_2$ *Assumed 0·5% lactic acid
Salami	11	3·3	70	NR*	5·0	NR	739	449 (319–631)	452 (315–647)	1175 (876–1582)	1115 (822–1512)	Trussell and Jemmi 1989	pH fell to 2·5 and NaCl increased to >5% by the end of the ripening period *Assumed lactic acid value of 0·5%
		20				NR	2480	1489 (1114–1989)	1542 (1135–2097)	1331 (985–1800)	1264 (927–1721)		

Brain heart infusion broth	28	12·6 13·7 16·7	0	0	7·4	Scott A	918 584 347	955 (662–1376) 858 (592–1245) 586 (377–911)	928 849 606	858 (611–1206) 783 (561–1093) 564 (387–822)	913 (653–1278) 840 (598–1168) 607 (413–892)	Miller 1992	
Acidified ground beef	37	3·2	0	0·28 0·33 0·53 1·03 1·79	5·1 5·0 4·7 4·3 4·0	Scott A, V7, HO-V	1276 518 274 127 12	315 (236–422) 279 (210–370) 175 (133–229) 66 (49–88) 21 (15–30)	277 (206–374) 259 (193–348) 196 (149–258) 95 (73–124) 32 (22–45)	232 (172–313) 207 (154–277) 135 (102–177) 57 (43–76) 21 (15–31)	189 (139–256) 177 (131–238) 140 (106–185) 84 (64–111) 37 (25–54)	Whiting and Masana 1994	*Assumed 20% residual of initial $NaNO_2$ levels
	37	3·2	30*	0·28 0·33 0·53 1·03 1·79	5·1 5·0 4·7 4·3 4·0		118 480 317 118 26	168 (125–227) 148 (111–197) 91 (69–119) 34 (26–44) 11 (8–16)	148 (109–200) 137 (101–184) 100 (76–133) 47 (37–61) 16 (11–22)	239 (176–323) 195 (146–260) 121 (93–158) 46 (35–61) 15 (11–22)	180 (133–243) 167 (124–224) 125 (95–165) 66 (51–86) 25 (18–36)		
Cottage cheese***	5	NR*	0	NR*	5·05	Scott A	2808**	3062 (1910–4908)	3137 (1928–5102)	1411 (890–2237)	1380 (865–2201)	Piccinin and Shelef 1995	*Assumed 1% NaCl and 0·5% lactic acid **Estimated by extrapolation of 576 h inactivation curve ***Also contained sorbic acid
Cottage cheese	5	NR*	0	NR*	4·89	Scott A	6980**	2618 (1668–4109)	2745 (1716–4391)	1360 (838–2016)	1296 (827–2030)	Piccinin and Shelef 1995	*Assumed 1% NaCl and 0·5% lactic acid **Estimated by extrapolation of 576 h inactivation curve
Reduced calorie mayonnaise	24	2·8	0	0·1* 0·3* 0·5* 0·7*	4·3 4·3 4·1 3·9	Scott A, V7, LM101M, LM103M, LM1087M, MF9904P635	204 123 95 70	784 (623–985) 622 (510–759) 385 (321–463) 233 (193–280)	651 (523–812) 584 (477–716) 393 (325–475) 239 (196–292)	589 (466–743) 477 (389–584) 321 (266–387) 212 (175–256)	447 (359–556) 424 (346–520) 333 (275–404) 243 (197–299)	Glass and Doyle 1991	*Acetic acid was the acidulant Also contained 0·1% potassium sorbate
Cholcaterol–free reduced calorie mayonnaise	24	2·3	0	0·3* 0·7*	4·0 3·9	Scott A, V7, LM101M, LM103M, LM1087M, MF9904P635	100 36	414 (339–506) 229 (189–276)	389 (318–475) 234 (191–288)	355 (289–436) 210 (173–255)	330 (270–404) 243 (196–301)	Glass and Doyle 1991	*Acetic acid was the acidulant Also contained 0·1% potassium sorbate
Sandwich spread	27	6·1	0	2·0*	3·3	ATCC 11911, 11915, 15313, 43256, 43257	27	18 (14–24)	18 (14–24)	21 (16–27)	18 (14–25)	Erickson and Jenkins 1991	*Acetic acid was the acidulant
Mayonnaise	27	9	0	1·8*	3·9	ATCC 11911, 11915, 15313, 43256, 43257	27	62 (47–81)	75 (56–101)	54 (41–70)	80 (56–112)	Erickson and Jenkins 1991	*Acetic acid was the acidulant

*, **, ***, See 'Comments' column.

While this relationship is empirical, its association with both lactic acid and sodium nitrite suggests that it may be related to the underlying mechanisms of action for antimicrobials that are dependent on a weak acid being present in an undissociated form. It also suggests that for such compounds, it would be better to calculate the 'minimum inactivation acceleration concentration' using the undissociated acid.

The usefulness of the four models is ultimately dependent on validating their ability to predict the non-thermal inactivation of *L. monocytogenes* in actual food systems. As an initial assessment, t_{4D} values were estimated from reported studies on the inactivation of *L. monocytogenes* and compared with values predicted by the four models (Table 7). This comparison includes a range of *L. monocytogenes* strains in a variety of foods and microbiological media.

Ideally, the models should consistently predict t_{4D} values that are as long or longer than the actual value. In most instances, this was the case when the prediction and its 95% confidence interval were considered. In general, models 1 and 2 performed somewhat better than models 3 and 4, possibly reflecting the fact that estimating residual nitrite content is difficult. A residual sodium nitrite value of 20–30% was assumed for the purposes of this comparison. While the current models were developed specifically for the use of lactic acid as the acidulant, several studies that involved the use of acetic acid were included in Table 7. The models performed reasonably well in these instances, predicting somewhat conservative t_{4D} values.

This approach to model validation has severe limitations. Often pertinent characteristics that can increase or decrease the rate of inactivation were not reported and had to be assumed (Table 7). For example, lactic acid concentrations were often not reported, and it was often unclear if investigators had cultured *L. monocytogenes* in a manner that maximized its acid tolerance (Kroll and Patchett 1992; Farber and Pagotto 1992; Buchanan *et al.* 1994). Furthermore, most investigators did not provide estimates of the variability associated with the reported inactivation rates. The techniques used by investigators to estimate inactivation rates must also be reviewed carefully. For example, the estimated t_{4D} times reported for labneth, a fermented dairy product (Gohil *et al.* 1996), were consistently lower than those predicted by the models (Table 7). However, the inactivation data were based on direct plating on a selective medium that would not have detected acid-injured cells. Comparison with literature values is further complicated by the fact that foods often contain additional antimicrobial components that are not considered in the models (e.g. sorbic acid). In most instances, additional factors accelerate inactivation so that the models actually provide a margin of safety. However, in some instances this might not be the case. For example, the presence of moderate levels of sodium chloride tends to protect *L. monocytogenes*. The use of a second humectant in combination with sodium chloride could increase or decrease inactivation rates depending on the final water activity and whether it was a 'permissive solute' (i.e. a solute that can be accumulated intracellularly with minimal impact on the cell's metabolic systems).

A second interesting example is the study by Whiting and Masana (1994) where the direct addition of lactic acid to ground beef was used to study the survival of *L. monocytogenes*. While the models correctly predicted the general effects of pH and lactic acid concentration, they consistently underestimated the observed t_{4D} values. This was surprising considering that the models provided good estimates of *L. monocytogenes* survival for several studies with fermented meat products. This suggests that the method of acidification may have an influence, the direct addition of lactic acid for control of pathogens not being as effective as its generation *in situ* during fermentation.

Overall, the expanded models appear to perform well as a means of rapidly estimating the survival of *L. monocytogenes* under a broad range of environmental conditions that lead to the non-thermal inactivation of the organism. In general, models 1 and 2 proved most effective when compared against inactivation rates reported in the literature, with model 1 being the easier to use in relation to variables that can be readily measured. However, models 3 and 4 may offer specific advantages when dealing with products where there is a need to more accurately estimate the interaction of pH and sodium nitrite concentration. In their current state, the models should provide reasonable estimates of the order of magnitude of the t_{4D} value for a variety of foods, and how the five variables interact to influence *L. monocytogenes* survival. In using such models, it is important to consider the variability associated with the predictions and include appropriate confidence intervals. Predictions of survival should be limited to $\leqslant$ 4300 h since that was the limit of the experimental trials upon which the models were developed. Extrapolation beyond that limit must be done with extreme care. As with any model, the use of these models for other than initial estimates should be predicated on their validation for the specific product of interest.

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Characterization of strains of *Leuconostoc mesenteroides* by analysis of soluble whole-cell protein pattern, DNA fingerprinting and restriction of ribosomal DNA

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F. VILLANI, G. MOSCHETTI, G. BLAIOTTA AND S. COPPOLA. 1997. Of 215 leuconostocs isolated from field grass, natural whey cultures and water-buffalo milk, 178 were identified as *Leuconostoc mesenteroides* ssp. *mesenteroides* while 37 strains could not be identified. Biochemical characterization allowed seven groups to be defined. Representative strains of each group and different habitat and nine reference strains were selected for further analyses. Protein profiles appeared suitable for species discrimination, but did not differentiate between the three subspecies of *Leuc. mesenteroides*. The technique also showed some differences among equivocal strains. DNA fingerprinting for most strains of *Leuc. mesenteroides* ssp. *mesenteroides* examined showed a different restriction pattern from that of the type strain. Ribotyping was not useful for discriminating species and subspecies of the genus *Leuconostoc*: *Leuc. mesenteroides* ssp. *mesenteroides* and ssp. *dextranicum* showed the same ribopattern as *Leuc. lactis* while *Leuc. mesenteroides* ssp. *cremoris* exhibited a pattern distinct from all the other species examined. On the basis of ARDRA-PCR, two main groups could be distinguished: the larger group included *Leuc. mesenteroides*, *Leuc. lactis*, *Leuc. pseudomesenteroides* and some unidentifiable strains; the second one included *Leuc. citreum*, *Leuc. fallax*, *Weissella paramesenteroides* and some unidentified strains.

INTRODUCTION

The genus *Leuconostoc* (*Leuc.*) includes Gram-positive, catalase-negative micro-organisms with irregular coccoid morphology. Their distinction from gas-forming heterofermentative lactobacilli has long been controversial. In fact, the two genera are now considered to be phylogenetically intermixed (Stackebrandt *et al.* 1983; Stackebrandt and Teuber 1988).

Garvie (1981) defined the salient characteristics of the genus *Leuconostoc* and recognized four species: *Leuc. mesenteroides* (with the three subspecies, *dextranicum*, *cremoris* and *mesenteroides*), *Leuc. paramesenteroides*, *Leuc. lactis* and *Leuc. oenos*, recently reclassified as *Oenococcus oeni* (Dicks *et al.* 1995). Since 1989 six further species have been described: *Leuc. carnosum* and *Leuc. gelidum* (Shaw and Harding 1989); *Leuc. pseudomesenteroides* and *Leuc. citreum* (Farrow *et al.* 1989), also known as *Leuc. amelibiosum* (Schillinger *et al.* 1989; Takahashi *et al.* 1992); *Leuc. argentinum* (Dicks *et al.* 1993); and an atypical leuconostoc named *Leuc. fallax* (Martinez-Murcia and Collins 1991). More recently, Collins *et al.* (1993) proposed that *Leuc. paramesenteroides* and related species be reclassified in a new genus, *Weissella*.

Comparative analyses of 16S and 23S rDNA sequences revealed the presence of three distinct lines of descent: *Leuconostoc sensu stricto*, the *Leuc. paramesenteroides* group (*Weissella*) and the species *Leuc. oenos* (Yang and Woese 1989; Martinez-Murcia and Collins 1990, 1991; Collins *et al.* 1993; Martinez-Murcia *et al.* 1993).

Leuconostocs are widespread in the natural environment, though probably because of their weak acidifying activity, they are not widely investigated. Apart from some spoilage roles, they are generally considered as flavouring agents in dairy, wine and vegetable fermentations. Their recognized antagonistic or synergistic properties in mixed microflora